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Synaptic Mechanisms of Induction and Maintenance of Long-Term Sensitization

Memory in *Aplysia*

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Abstract

The marine snail *Aplysia californica* exhibits a simple defensive withdrawal reflex that can undergo several forms of learning. In particular, the reflex can exhibit long-term sensitization (LTS), a form of nonassociative memory. LTS is mediated by long-term facilitation (LTF) of the monosynaptic connection between the sensory and motor neurons that mediate the withdrawal reflex. LTS and LTF represent one of the best-understood model systems of long-term memory extent. Furthermore, discoveries from work on this system have provided fundamental insights into the cellular and molecular mechanisms that mediate the induction and maintenance of long-term memory. The present chapter reviews this work; it concludes with a discussion of recent studies of the role of protein kinase M in the persistence of the long-term memory and of memory reconsolidation in *Aplysia*. It is suggested that the study of LTS and LTF can provide important mechanistic information these two intriguing memory phenomena.

Introduction

One of the more remarkable features of the brain is its ability to transform experience into memories that persist for a lifetime. That memories can remain more or less stable over the course of many decades despite the significant physical changes that occur in the brain with age is one of the grand mysteries of neuroscience. The last 30 years have witnessed rapid progress in our understanding of how long-term memories are induced in the brain; by comparison, we understand far less about how memories are maintained.

Much of what we know about the cell biology of long-term memory has come not from studies of the mammalian brain but, rather, from studies of the nervous systems of invertebrate organisms, the most influential of which, arguably, are those using the marine snail *Aplysia californica*. Why this relatively humble mollusk should have played such a key role in mechanistic studies of long-term memory requires some explanation. *Aplysia* has several important features that facilitate relating cellular and molecular changes to learned behavioral changes. First, its central nervous system (CNS) possesses only about 20,000 neurons ¹, compared to, for example, about 21 million in the brain of a rat ². This greatly simplifies the task of identifying learning-related sites of change in the nervous system. The logic here is similar to that employed by the legendary Spanish neuroanatomist, Ramon y Cajal, who chose the embryos of animals such as dogs and cats for his initial investigations of the fine structure of the brain using the Golgi stain. In justifying this approach Cajal ³ stated, “Since the full grown forest [i.e., the adult brain] turns out to be impenetrable and indefinable, why not revert to the study of the young wood, in the nursery stage, as we might say?” Similarly, the simplicity of the *Aplysia* nervous system renders the specific pattern of cellular changes produced by a learning

experience more readily visible in *Aplysia* than in the relatively impenetrable neural thicket of the mammalian brain. Another major advantage of *Aplysia* is that neurons that mediate specific behaviors can be identified, and then individually dissociated and placed into dissociated cell culture. This means that behaviorally relevant neural circuits can be recreated in vitro. Importantly, these in vitro circuits have been shown to exhibit most, if not all, of the same forms of synaptic plasticity that they exhibit in vivo. This feature has been extensively exploited, most prominently by Eric Kandel and his colleagues, with the result that arguably more is known about the cell biology of learning and memory in *Aplysia* than in any other organism ⁴. The monosynaptic connection between the sensory and motor neurons that mediate the defensive withdrawal reflex of the gill and siphon (the sensorimotor synapse) has been a particularly fruitful system for mechanistic analyses of learning. Among the major contributions of research on the sensorimotor synapse is an understanding of the critical role in long-term, learning-related synaptic plasticity of protein synthesis and gene transcription ⁵, particularly that stimulated by the transcription factor cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) ⁶⁻⁹. Importantly, these processes have each been found to play a critical role in mammalian learning-related long-term synaptic plasticity as well ¹⁰⁻¹³, which indicates that there has been broad conservation of the cellular and molecular mechanisms of learning and memory over the course of evolution.

The present review will focus on the one specific form of long-term memory in *Aplysia*, the memory for long-term sensitization (LTS) of the defensive withdrawal reflex ¹⁴. Arguably, knowledge regarding underlying mechanisms has advanced further for LTS than for any other form of long-term memory in *Aplysia*. The review will begin with a

description of the cellular and molecular mechanisms that mediate induction of LTS. It will then describe what is known about the mechanisms that underlie the maintenance of LTS. Finally, future directions toward an understanding of this form of nonassociative learning and memory will be discussed. Although sensitization in *Aplysia* involves nonsynaptic, particularly changes in neuronal excitability, this chapter will focus on the synaptic mechanisms of LTS. (For a recent review on nonsynaptic mechanisms of learning in *Aplysia* and other organisms, see ref. [15](#).)

Long-Term Sensitization in *Aplysia*: Mechanisms of Induction

When an *Aplysia* receives a noxious stimulus, such as an electrical shock to its tail, the endogenous monoaminergic transmitter serotonin (5-HT) is released into the animal's CNS from a network of central serotonergic neurons [16-19](#). This release of 5-HT triggers a number of cellular changes that lead to enhancement of the defensive withdrawal reflex [20](#). Much of the analysis of these sensitization-related cellular changes has focused on the monosynaptic connections between the central sensory and motor neurons that mediate the reflex. Two such connections that have been particularly well studied with respect to sensitization have been that between the sensory and motor neurons that mediate contraction of the gill and siphon [21-23](#); and the synaptic connection between the pleural sensory neurons and the pedal motor neurons, which mediates contraction of the tail [24,25](#). The central sensory and motor neurons that mediate the gill- and siphon-withdrawal reflex are located in the abdominal ganglion [26](#). Tail withdrawal is mediated by sensory neurons in the paired pleural ganglia, whereas the motor neurons that mediate this reflex are located in the paired pedal ganglia [27](#).

Tail (or tail nerve) shock and 5-HT facilitate transmission at the sensorimotor synapse^{25,28-32}. (Other endogenous transmitters can produce facilitation of the sensorimotor synapse, particularly the small cardioactive peptides [SCPs]^{30,33} and nitric oxide, released by the L29 facilitatory interneurons³⁴; however, thus far only 5-HT has been shown to support long-term facilitation of the sensorimotor synapse.) The facilitation of the sensorimotor synapse plays a prominent role in both short-term^{31,35} and long-term³⁶ sensitization.

A single tail shock, or a single, brief (≤ 5 -minute) pulse of 5-HT will produce only short-term (< 30 minutes) behavioral and synaptic enhancement. However, several, spaced tail shocks, or repeated application of 5-HT, can result in long-term (≥ 24 hours) behavioral and synaptic changes in *Aplysia*.^{5,14,36,37} The long-term memory for sensitization differs in several fundamental respects from the short-term form. First, the long-term memory, unlike short-term form, depends on protein synthesis and gene transcription^{5,7-9,38-48}. Second, LTS, unlike short-term sensitization (STS), typically—although not invariably⁴⁹—involves significant structural reorganization, particularly the growth of new presynaptic varicosities⁵⁰⁻⁵⁶.

Comment on cellular locus of inductive processes

A complication in describing the cellular and molecular mechanisms that underlie the induction—as well as the maintenance—of LTS in *Aplysia* is identifying the cellular site where the mechanisms operate. For decades it was assumed that the changes mediating the long-term behavioral and synaptic changes that characterized LTS were confined to the presynaptic sensory neuron^{4,57}. Although, as summarized below, there are indeed

important presynaptic changes that contribute to the induction of the long-term memory for sensitization, it is now apparent that such changes are insufficient to account for the memory, and that postsynaptic changes are also critical (for review see ref. [58](#)). Furthermore, in at least some cases the changes, whether presynaptic or postsynaptic, are not cell autonomous, but require as yet unidentified transsynaptic signals; these signals may be retrograde [59-61](#) or anterograde [62](#). The role of postsynaptic mechanisms and transsynaptic signals in long-term memory in *Aplysia* has been slighted (but see refs. ^{61,63-65}) prior to the last decade. There have been far more studies of presynaptic changes that underlie long-term memory than of postsynaptic changes; biochemical and molecular studies of LTS have been particularly weighted toward presynaptic mechanisms. A cursory survey of the literature on LTS in *Aplysia* might therefore lead the naïve reviewer to conclude that presynaptic mechanisms play a larger role than postsynaptic mechanisms in this form of learning. But the present prominence of presynaptic mechanisms in studies of long-term memory in *Aplysia* is unlikely to reflect any underlying biological reality [58](#), and the role of postsynaptic biochemical and molecular changes in LTS represents a rich, nearly untapped, vein of discovery for future investigation.

Biochemical and molecular mechanisms of induction

5-HT, released into the CNS of *Aplysia* by sensitizing stimuli, such as tail shock [17](#), activates the second messenger cAMP within sensory neurons ^{28,66-71}. Repeated or prolonged application of sensitizing stimuli, or of 5-HT, produces prolonged elevation of cAMP within the sensory neurons [70](#), resulting in persistent activity of protein kinase A (PKA) [72](#). The persistent activity of PKA, a heterodimer with two regulatory and two

catalytic subunits, results, at least in part, from a selective downregulation of the regulatory subunits of the protein kinase ⁴⁵. The decrease in the ratio of regulatory to catalytic subunits requires protein synthesis ⁴⁵ and is caused by selective proteolysis of the regulatory subunits ⁷³.

The loss of PKA's regulatory subunits and its consequent persistent activation, produced by repeated stimulation with 5-HT, can lead to a translocation of the catalytic subunits from the cytoplasm of the sensory neuron to its nucleus ⁶⁷. The nuclear translocation of the PKA catalytic subunits, in turn, activates cyclic AMP response element binding (CREB) protein, a transcriptional activator ^{7,8,74}; the activation of CREB is a key step in the induction of long-term memory in *Aplysia*. CREB represents a family of transcription factors ⁷⁵. In *Aplysia* the activator isoform is termed CREB1. The activity of CREB1 is normally inhibited by a repressor CREB isoform, known as CREB2 ⁴¹; stimuli that induce LTS or long-term facilitation (LTF) cause the derepression of CREB1 by CREB2, thereby leading to CRE-dependent gene expression ⁶. It is believed that this derepression is mediated by extracellular signal-regulated kinase (ERK)/mitogen-activated kinase (MAPK); cytoplasmic MAPK is activated within sensory neurons by prolonged 5-HT treatment and translocates to the nucleus where it phosphorylates CREB2 ⁷⁶⁻⁸⁰. Among the genes stimulated by CREB1 are the immediate-early gene ubiquitin-C hydrolase ⁸¹, which encodes an enzyme that enhances proteasome activity ⁸², as well as genes that encode two other transcription factors, *Aplysia* Activating Factor (ApAF) ⁸³ and the CCAAT- box-enhanced binding protein (C/EBP) ⁸⁴. ApAF and C/EBP, together, possibly, with other as yet unidentified, trigger a second wave of gene activation; one of the consequences of this later wave of transcription is believed to be

the growth of new synaptic connections structural growth^{53,85} (see below).

Presynaptic mechanisms of LTS and LTF

LTS is associated with facilitation of the monosynaptic connection between the central sensory and motor neurons that mediate the siphon- and tail-withdrawal reflex^{36,37,86}.

Because short-term facilitation (STF) of the sensorimotor synapse is largely, perhaps exclusively, the result of presynaptic changes^{29,87-89}, it was seductive to extend the presynaptic model to LTF. Unquestionably, LTF results partly from enhanced presynaptic release of glutamate, the neurotransmitter used by *Aplysia* sensory neurons⁹⁰⁻⁹³.

Nonetheless, the idea that LTF results exclusively^{4,57,87}, or even predominately, from presynaptic mechanisms is no longer tenable^{58,59,62,94}.

A major advance in the mechanistic analysis of LTF in *Aplysia* was the demonstration of LTF in sensorimotor cocultures by Montarolo et al. in 1986⁵. The methodology of Montarolo et al. has been used to make many of the most important discoveries regarding long-term memory in *Aplysia*. For in vitro studies of long-term synaptic plasticity identified sensory and motor neurons are individually dissociated from central ganglia of *Aplysia* and placed into cell culture together (**Figure 1B**). Under the right conditions the neurons will form chemical synaptic connections; the strength of these connections gradually increases over the first several days in vitro, and then reaches a stable value, typically after 3-5 days. (The precise time at which the synapses stabilize in strength stabilizes depends on factors such as the specific identities of the pre- and postsynaptic neurons—for example, whether the giant gill- and mantle- motor neuron L7^{1,5} or one of the small siphon motor neurons^{23,95} is used for the postsynaptic neuron—and

the condition of the animals from which the neurons were dissociated or the cell culture medium used.) After the synaptic strength has stabilized, as indicated by measurements of the excitatory postsynaptic potential (EPSP), the experiment is begun. LTF is induced by five spaced pulses of 5-HT; each pulse is typically 5 min long and separated from the other pulses by a 15 min period during which the drug is washed out of the culture dish (**Figure 1B**). This training protocol, referred to as the 5X5-HT protocol, was originally designed to mimic the spaced delivery of tail shocks used to induce behavioral LTS. (If activation of the sensory neuron is paired with the 5-HT pulses the result is an associative form of LTF^{96,97}. Activity-dependent LTF, which mediates so-called “site-specific” sensitization^{98,99} and also plays a role in classical conditioning of the withdrawal reflex¹⁰⁰⁻¹⁰³, differs somewhat mechanistically from activity-independent LTF¹⁰⁴.) Training with the 5X5-HT protocol can induce LTF that persists for 24 hr⁵ to 72 hr^{105,106}. Commonly, the 5-HT is simply added to the cell culture dish, but in some studies the 5-HT has been locally perfused in spaced bouts over a region of sensorimotor synaptic contact in order to induce synapse-specific LTF^{105,107}. (Although the 5X5-HT delivery method, which uses a uniform interval between the pulses of 5-HT, is certainly effective, it has recently been shown that a protocol involving non-uniform interstimulus intervals results in enhanced LTF¹⁰⁸.)

LTF, like LTS itself, requires protein synthesis and CREB-dependent gene transcription^{5,7,8,41,109}. Both presynaptic^{45,105,107,110} and postsynaptic^{59,62,111} protein synthesis are involved, and a critical component of this LTF-related protein synthesis is local^{42,105,107,110-113}. Among the local mRNAs that are translated by prolonged 5-HT stimulation are those that encode the cytoskeletal proteins, α 1-tubulin and β -thymosin, which may

play roles in synaptic growth, and ribosomal proteins ¹¹². The latter may increase the number of translationally competent ribosomes at the stimulated synapses. By thereby producing a localized site for translation, this mechanism could serve to restrict the products of learning-related transcription to stimulated synapses; such a mechanism may help to account for synapse-specificity during learning ^{42,107,114}.

One important function of local protein synthesis during LTF is to stimulate a retrograde signal that travels to the nucleus and activates gene transcription. This phenomenon was first described by Martin et al. ¹⁰⁷ in their study of synapse-specific LTF. In this study two sensory neurons were cocultured with, and synaptically connected to, a single L7 motor neuron. When one of the two regions of sensorimotor contact, but not the sensory neuron cell body, was given 5X5-HT training via local perfusion, the trained sensorimotor synapse exhibited LTF, whereas the second, untrained, synapse did not. By contrast, when local 5X5-HT treatment failed to induce LTF of the stimulated synapse when the protein synthesis inhibitor emetine was included in the 5-HT-containing solution. Martin and colleagues further showed that when a brief pulse of 5-HT—which, alone, produces only STF—was applied to one of the two regions of sensorimotor contact, and the other region of sensorimotor contact was given the 5X5-HT training, the result was that both synapses underwent LTF. This result implies that the 1X5-HT-trained synapse was able to “capture” the somal products required for LTF that are produced by the 5X5-HT treatment of the other synapse; in other words, the 1X5-HT treatment induces a “tag” of the synapse that is necessary, albeit not sufficient, for the synapse to undergo LTF. Induction of this tag does not depend on protein synthesis, because it is not prevented if the synaptic region is exposed to emetine (a long-lasting

protein synthesis inhibitor) together with the single pulse of 5-HT. Taken together, Martin et al.'s results indicate that local 5X5-HT training induces two processes required for LTF: a protein synthesis-dependent retrograde signal that activates gene transcription, and a protein synthesis-independent tag that permits the synapse to capture the somal proteins/mRNAs whose production results from the 5X5-HT stimulated transcription. (Note that similar results have been reported for long-term potentiation [LTP] in the mammalian hippocampus ^{115,116}.) In a study of sensorimotor synapses in pleural-pedal ganglia, Sherff and Carew also found evidence for a protein synthesis-dependent retrograde signal that can be induced by relatively brief 5-HT treatment and that supports the induction of LTF ¹¹⁷.

One presynaptic protein that is locally synthesized in response to prolonged 5-HT stimulation is the neuropeptide sensorin ¹¹⁸. Sensorin is peptide first identified in *Aplysia* where it found exclusively in sensory neurons ¹¹⁹. When a sensorimotor coculture is stimulated with multiple pulses of 5-HT there is a rapid, protein synthesis-dependent increase in sensorin expression in the presynaptic varicosities of sensory neurons, as assessed with immunohistochemistry ¹²⁰⁻¹²². Interestingly, this increase in local translation of sensorin requires a postsynaptic signal; the increase does not occur if the motor neuron is injected with the rapid Ca^{2+} chelator 1,2-bis(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid (BAPTA), or in the varicosities of isolated sensory neurons stimulated with 5X5-HT ^{59,60}. The enhanced local expression of sensorin is followed by release of the neuropeptide from the varicosities ^{122,123}; released sensorin binds to autoreceptors on the sensory neuron, thereby activating presynaptic MAPK, which then translocates to the sensory neuron's nucleus ¹²³. Release of sensorin is necessary for LTF: treatment of

sensorimotor cocultures with an anti-sensorin antibody blocks the induction of LTF ¹²³. Furthermore, release of sensorin requires PKA activity. ([Note that it also requires protein kinase C activity. See ref. ^{104, and below}](#).) This finding has led Schacher and colleagues to propose that sensorin release and binding to autoreceptors is a necessary intermediate step between the activation of PKA and the activation and nuclear translocation of MAPK ^{122,123} during LTF.

Besides inducing an increase in local synthesis of sensorin, multiple pulses of 5-HT cause an increase in transcription of sensorin ¹¹⁸; the sensorin mRNAs are exported to synapses in sensorimotor cocultures, and this synaptic localization of the mRNAs requires an appropriate target motor neuron ^{118,124,125}. This result addresses a crucial general question for molecular models of long-term memory, namely, whether the products of nuclear transcription can support synapse specificity during learning-related long-term synaptic plasticity ¹²⁶. Evidence from studies of long-term potentiation in the mammalian hippocampus and hippocampal-dependent learning suggest that mRNAs can indeed be targeted to active synaptic regions ¹²⁷⁻¹²⁹. Martin and colleagues have shown that the synaptic localization of sensorin reporter mRNAs in *Aplysia* sensorimotor cocultures is accomplished through cis-acting localization elements (LEs). LEs within the 3' UTR are sufficient for localization of the reporter mRNA to distal sensory neurites, whereas LEs within the 5' UTR are required for synaptic localization of the reporter mRNAs ¹²⁵. Recently this group has identified a 66 nucleotide-long element in the 5' UTR of sensorin that is both necessary and sufficient for synaptic localization of the mRNA ¹³⁰.

Structural changes underlying long-term sensitization memory

Early ultrastructural work in intact animals showed that LTS is associated with presynaptic morphological changes. In particular, the induction of LTS in *Aplysia* results in an increase in the number of presynaptic varicosities, the number of active zones associated with sensory neurons terminals, and in the number of vesicles per active zone ^{50,51,131}. Furthermore, generally, there is enhanced outgrowth of the neurites of sensory neurons during LTS ^{37,51,132}, although some training protocols that induce LTS do not induce outgrowth of sensory neurites ⁴⁹.

Glanzman et al. extended these results to the in vitro system; they demonstrated that LTF results in an increase in both the number of presynaptic varicosities and in the number of sensory neurites in sensorimotor cocultures ⁶¹. Since then, much of the work on structural changes associated with long-term, sensitization-related memory in *Aplysia* has used the in vitro system. The increase in presynaptic growth in *Aplysia* sensory neurons due to LTF requires protein and RNA synthesis ¹³³, can be induced by injecting cAMP into the sensory neuron, and is mediated by CREB1 ¹⁰⁷. Just as is the case for LTS, however, there are training protocols that can induce LTF that do not produce the presynaptic outgrowth ⁴², although the facilitation induced by these protocols does not persist for significantly longer than 24 hours.

One of the requirements for the structural changes that typically accompany LTS is the downregulation of an *Aplysia* neural cell adhesion molecule (ApCAM). 5X5-HT stimulation causes a downregulation of the ApCAM protein in sensory neurons; it also leads to a decrease in the number of ApCAM molecules on the surface of sensory neurites ¹³⁴. The decrease in the amount of surface ApCAM molecules results from their

endocytosis ⁵⁴. Furthermore, applying an antibody to ApCAM to isolated sensory neurons causes their neurites to defasciculate ¹³⁴; such defasciculation may well be a critical step in the 5HT-induced outgrowth of sensory neurites. The down-regulation of presynaptic ApCAM expression, and the internalization of surface ApCAM molecules on sensory neurites and subsequent neuritic defasciculation are likely to be initial steps in the process of structural change that accompanies LTS and LTS ^{135,136}.

Besides playing a critical role in the restructuring of sensory neurons during long-term learning in *Aplysia*, internalization of apCAM also subserves retrograde synapse-to-nucleus signaling. Lee et al. ⁷⁴ found that apCAM is associated with a transcriptional activator, CAM-associated protein (CAMAP). In its non-activated state CAMAP is bound to the cytoplasmic tail of apCAM molecules in the cell membrane of the sensory processes. Repeated pulses of 5-HT stimulate PKA, which phosphorylates CAMAP; phosphorylation of CAMAP, in turn, causes its dissociation from apCAM, an essential step in the internalization of the apCAM {Mayford, 1992 #42;Bailey, 1992 #4920}. The freed CAMAP then translocates to the nucleus where, it serves as a coactivator, together with CREB1, of the downstream transcription factor CCAAT enhancer-binding protein (C/EBP), an immediate gene whose activation is critical for the consolidation of LTF ⁸⁴.

Whereas the majority of the work on structural changes related to long-term memory in *Aplysia* has focused on presynaptic changes, LTS also involves postsynaptic structural changes. In an electron microscopic study Bailey and Chen ⁶³ quantified changes in the number of spine-like filapodia on the neurites of the identified motor neuron L7 that occurred in intact animals following LTS-inducing training. They observed an increase in both the number of small ($< 0.5 \mu\text{m}$), spine-like L7 processes.

Furthermore, they also saw an increase in the number of presynaptic contacts per square micrometer of L7's dendritic surface. These results suggest that LTS causes a coordinated growth of pre- and postsynaptic structures, resulting in increased synaptic contact between the sensory and motor neurons.

Postsynaptic and transsynaptic mechanisms

As pointed out above, persistent memory in *Aplysia* involves postsynaptic, as well as presynaptic changes. One such change is an increase in postsynaptic Ca^{2+} . Cai et al. ⁵⁹ reported that LTF of the in vitro sensorimotor synapse is blocked by an injection of the rapid chelator BAPTA into the motor neuron prior to 5-HT stimulation. This result implies that multiple pulses of 5-HT cause a rise in postsynaptic intracellular Ca^{2+} . Similar results have been obtained if a postsynaptic injection of BAPTA is made prior to a 10-minute exposure to 5-HT ^{88,89}, which causes intermediate-term facilitation (ITF), that is, facilitation that persists from 30 minutes to approximately 3 hours after the exposure to 5-HT ¹³⁷. The 5-HT-induced rise in postsynaptic Ca^{2+} results from release from intracellular stores, both inositol-1,4,5-trisphosphate (IP_3)-mediated and ryanodine receptor-mediated stores ^{88,138}. The increased intracellular Ca^{2+} activates postsynaptic calcium-calmodulin II (CaMKII) ⁸⁹. The downstream effects of this pathway's activity remain to be determined, but are likely to involve at least some of those described below.

Prolonged 5-HT treatment in both the intact nervous system ⁶⁵ and sensorimotor cocultures ⁶⁴ produces functional upregulation of α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-type glutamate receptors (AMPA receptors). The early evidence for this effect came from studies in which the effect of brief pulses of AMPAR agonists

delivered to *Aplysia* motor neurons was quantified electrophysiologically before and after prolonged exposure to 5-HT. It was found that this treatment resulted in a greater evoked potential in the motor neurons 24 hours later ^{64,65}. A 10-minute-long, ITF-inducing, exposure to 5-HT has a similar effect ^{88,138}. Together, these results imply that prolonged 5-HT stimulation triggers an increase in the number of postsynaptic AMPARs.

Investigations of mammalian LTP and learning have shown long-term synaptic plasticity and learning are mediated, in part, by modulation of AMPAR trafficking ^{139,140}. In the case of the mammalian CNS, modulation of AMPAR receptor trafficking can be initiated by activation of postsynaptic *N*-methyl-D-aspartate receptors (NMDARs). The evidence from the work on *Aplysia* suggests that a monoamine can similarly initiate modulation of AMPAR trafficking. In mammals activation of NMDARs causes additional AMPARs to be inserted into dendrites, either at extrasynaptic sites or directly into the postsynaptic membrane ¹⁴⁰. The extrasynaptically inserted AMPARs then diffuse into the synapse ¹⁴¹. Direct insertion of the AMPARs is believed to be mediated by exocytosis from a recycling pool of receptors ^{142,143}.

The evidence from studies of ITF and LTF in *Aplysia* supports the following scheme: 5-HT exposure causes an increase in postsynaptic Ca^{2+} due to release from intracellular stores; the increased intracellular Ca^{2+} then drives insertion of additional AMPA into postsynaptic sites via an exocytotic mechanism ^{59,62,88,138}. This could involve direct synaptic insertion from a recycling pool of vesicles or lateral diffusion following exocytotic insertion into extrasynaptic sites. Unfortunately, at present there are no antibodies to *Aplysia* AMPARs available and, therefore, one cannot directly monitor possible movement of endogenous AMPARs into and out of postsynaptic sites. On the

other hand, several *Aplysia* AMPA-type receptors have been cloned ^{94,144}, which has permitted experimenters to fabricate exogenous constructs of green fluorescent protein (GFP)-tagged *Aplysia* AMPA-type receptors and express these constructs in *Aplysia* motor neurons. Using this method, together with fluorescence confocal microscopy, the experimenters monitored the effect of 5-HT treatment on the exogenous AMPA-type receptors. The resulting data are consistent with the idea that LTF ⁹⁴ and ITF ⁶² in *Aplysia* involve modulation of postsynaptic AMPAR trafficking. In the case of LTF the 5X5-HT training produced enhanced clustering of AMPA-type receptors in the motor neurons of sensorimotor cocultures ⁹⁴, whereas in the case of ITF a 10-minute exposure to 5-HT caused a rapid increase in the number of GFP puncta in the postsynaptic cell membrane, reflecting receptor insertion.

Dopamine causes the insertion of new AMPARs into the dendrites of hippocampal neurons, and this insertion depends on local protein synthesis ¹⁴⁵. Postsynaptic protein synthesis may also play a role in the functional upregulation of AMPA-type receptors caused by prolonged 5-HT stimulation in *Aplysia*. Although it was originally concluded that postsynaptic protein synthesis was not mechanistically involved in LTF ^{65,107}, recent work has established that proteins synthesized by the motor neuron are, in fact, critical for this form of synaptic plasticity ^{59,62}. Furthermore, at least some of these postsynaptic proteins result from local synthesis ¹¹¹. Besides mediating the increased number of postsynaptic AMPA-type receptors ⁶⁵, postsynaptic protein synthesis probably subserves other important mechanistic roles in LTF. For example, it is likely to mediate the growth of new postsynaptic spines ⁶³. Another potential role for protein synthesis is in the regulation of postsynaptic protein kinase C (PKC). Recent work has

implicated the constitutively active protein kinase M (PKM) fragment of the atypical *Aplysia* PKC isoform, named PKM Apl III ¹⁴⁶, in the maintenance of the memory for ITF ^{147,148}, as well as LTF and LTS (below) ¹⁴⁹. In *Aplysia* PKM Apl III is formed by Ca^{2+} -dependent proteolytic cleavage of PKC Apl III, the atypical isoform ¹⁴⁶. Bougie et al. ¹⁴⁸ used a Förster Resonance Energy Transfer (FRET) reporter construct to measure 5-HT-induced cleavage of PKC Apl III in *Aplysia* motor neurons. (Note that 5-HT does not induce cleavage of exogenous PKC Apl III in *Aplysia* sensory neurons ¹⁴⁸.) They observed that a 10-minute exposure to 5-HT caused cleavage of the atypical PKC, and this cleavage depended on calpain and protein synthesis. (Interestingly, the mammalian homolog of PKM Apl III, PKM ζ , is generated by local translation of the mRNA, which is formed by transcription from an alternate start site within the PKC ζ gene ¹⁵⁰.)

A fascinating theme to emerge from recent studies of learning-related persistent synaptic plasticity in *Aplysia* is the importance of transsynaptic mechanisms. The first evidence for a critical role for retrograde signaling in LTF came from the study of Glanzman et al. ⁶¹ of in vitro changes in presynaptic structure that mediate LTF. These investigators examined the effect of multiple pulses of 5-HT on the structure of isolated sensory neurons in culture and sensory neurons of sensorimotor cocultures. Whereas the 5-HT treatment triggered significant outgrowth of sensory processes and varicosities in the cocultures, it had no effect on the structure of isolated sensory neurons. These results suggest that a signal from the motor neuron is required for the 5-HT-stimulated growth of the sensory neuron. Later work established that LTF depended on postsynaptic Ca^{2+} , because it could be blocked by injecting BAPTA into the target motor neuron prior to the 5X5-HT treatment ^{59,62}. (Postsynaptic BAPTA also blocks the induction of ITF ^{88,89}.) This

result implies that at least some of the presynaptic changes associated with LTF do not result exclusively from sensory neuron-autonomous processes, but require a retrograde signal for their expression. In support of this idea, one critical presynaptic mechanism that mediates LTF, the synaptic translation of the sensory neuron peptide sensorin, has been shown to require Ca^{2+} signaling in the motor neuron ^{59,60}. A retrograde signal implicated in LTF is an interaction between the synaptic adhesion molecules neurexin and neuroligin ¹⁵¹. Choi et al. ¹⁵² identified *Aplysia* homologs of neurexin and neuroligin, ApNRX and ApNLG; ApNRX was found to be present in sensory neurons, whereas ApNLG was present in motor neurons. Recombinant versions of the two molecules were found to bind to each other. Furthermore, overexpression of ApNRX in sensory neurons and overexpression of ApNLG in motor neurons of sensorimotor cocultures produced a long-term increase in the strength of the sensorimotor synapse, while injection of antisense ApNRX in the presynaptic sensory neuron or antisense ApNLG in the postsynaptic motor neuron blocked the induction of LTF as well as the presynaptic structural changes associated with LTF. These results represent strong evidence for a role in LTF for transsynaptic signaling via a neurexin-neuroligin interaction; whether other transsynaptic pathways mediate retrograde signaling during LTF remains to be determined.

Recently, anterograde signaling has also been implicated in LTF in *Aplysia*. Jin et al. ¹⁵³ reported that spontaneous release of transmitter from the sensory neuron was required for the induction of ITF and LTF. Additionally, this group found that spontaneous presynaptic release activates postsynaptic the group I metabotropic receptor mGluR5; activation of mGluR5, in turn, elevates postsynaptic Ca^{2+} through release from

IP₃-mediated stores ⁶². This finding is consistent with previous evidence implicating release of Ca²⁺ from IP₃-mediated postsynaptic stores in ITF in *Aplysia* ^{88,138}.

Intriguingly, Jin et al. ⁶² found that injecting botulinum toxin, an exocytotic inhibitor, into presynaptic sensory neurons blocked the increase in AMPA-type receptors in the postsynaptic induced by a 10-minute application of 5-HT. These results suggest that modulation of postsynaptic AMPA-type receptor trafficking is regulated by an anterograde signal linked to enhanced presynaptic release.

The mechanistic model for LTS in *Aplysia* that has emerged within the previous decade is strikingly complex (**Figure 2**). Just a little over ten years ago it was widely believed that exclusively presynaptic mechanisms could adequately account for LTS and LTF ^{4,87}. Now, however, it is apparent that both pre- and postsynaptic changes are involved in the induction and stabilization of long-term memory; moreover, these changes are coordinated by retrograde and anterograde signaling. It is perhaps surprising that a simple, nonassociative form of learning in a relatively simple invertebrate organism has proved to involve such a rich repertoire of cellular and molecular processes. No doubt, there remain mediatory processes to be identified.

Maintenance of LTS Memory in *Aplysia*

Although significant progress has been made toward a mechanistic understanding of the induction and early stabilization of LTS (**Figure 2**), far less is understood about how the long-term memory persists within the nervous system of *Aplysia*. However, the previous decade has witnessed accelerated interest in memory maintenance in *Aplysia*, and we now have some insights into this fascinating problem.

CPEB and the prion hypothesis

One idea proposed to explain the persistence of the memory for LTF, and by extension, for LTS, centers on the translational activator cytoplasmic polyadenylation element binding protein (CPEB). CPEB mediates polyadenylation-induced translation and plays a key role in cell division during development ¹⁵⁴. A CPEB isoform has been identified in the nervous system of *Aplysia* (ApCPEB) ¹¹⁰; this isoform has several properties that make it attractive as a memory maintenance molecule in *Aplysia*. mRNAs of ApCPEB are present in the neurites of sensory neurons and translation of ApCPEB mRNA is stimulated locally within the neurites by a prolonged application of 5-HT ¹¹⁰. Another suggestive property of ApCPEB is that it can exist in two states, a monomeric state in which the protein is inactive, or acts as a repressor, and an active, multimeric state; in the multimeric state ApCPEB can recruit monomeric proteins to the multimer, and thereby become self-sustaining ¹⁵⁵. Thus, multimeric ApCPEB can act as a self-sustaining, local translational hub within sensory neurites, and thereby promote ongoing protein synthesis at the synapse. In this way ApCPEB could serve both as a synaptic marker and a mechanism for sustaining synapse-specific, long-term memories ¹⁰⁷. Consistent with this idea, 5X5-HT stimulation can induce mulimerization of ApCPEB in *Aplysia* sensory neurons, and injecting an antibody to multimeric ApCPEB into sensory neurons 24 hours after 5X5-HT treatment disrupts the maintenance of LTF ¹⁵⁵. Studies of long-term memory in *Drosophila* ^{156,157} provide further support for the involvement of CPEB in memory persistence.

The amyloid-like properties of ApCPEB in *Aplysia* have led to the suggestion that this molecule is a prion ^{155,158}. This is an intriguing idea, because it suggests a functional role for prions in the non-diseased brain, in contrast to its pathogenic role in devastating diseases such as Creutzfeldt-Jacob Disease ¹⁵⁹. Regardless of whether or not ApCPEB is a true prion, however, it is unlikely to mediate the maintenance of long-term memory in *Aplysia* beyond the first two days. Evidence for this assertion comes from a study by Miniaci et al. ¹⁰⁵, who examined the effect on the memory for LTF of inhibiting protein synthesis at various times after treating sensorimotor cocultures with 5X5-HT. Consistent with a role for ApCPEB in the early maintenance of long-term synaptic memory, treatment with the protein synthesis inhibitor emetine at 24 hours or 48 hours after 5-HT training disrupted LTF, as indicated by tests at later times; by contrast, emetine treatment at 72 hours after 5-HT training did not affect LTF. Similar results were obtained from local application of an antisense ApCPEB oligonucleotide to sensorimotor synapses. Thus, by 72 hours after training, local translation by ApCPEB would appear not to be critical for the maintenance of LTF.

PKM and the maintenance of long-term memory in *Aplysia*

Work in mammals has implicated the constitutively active fragment of atypical PKC ζ , PKM ζ , in the persistence of long-term synaptic plasticity and memory ¹⁶⁰⁻¹⁶⁴. As mentioned above, the nervous system of *Aplysia* contains an isoform of PKM ζ , PKM Apl III. This kinase can be inhibited by the zeta inhibitory peptide (ZIP), which encodes the amino acid sequence of the pseudosubstrate region of the inhibitory domain of the atypical

PKC isoform, PKC Apl III ¹⁴⁶, as well as, at micromolar concentrations, by the PKC inhibitor chelerythrine ¹⁴⁷. We have used these two compounds to test for a role for PKM Apl III in LTS and LTF in *Aplysia*. We have found that treatment with either ZIP or chelerythrine disrupts the maintenance of LTS ¹⁴⁹. (In our experiments the drugs were injected directly into the hemolymph of the animals at various times after sensitization training with tail shocks.) Zip or chelerythrine eliminated the memory for LTS, even when applied as late as 7 days after training; furthermore, the memory did not reappear for at least 2 days after the drug injection, nor could it be reinstated by a brief bout of sensitization training. Injection of the scrambled ZIP peptide did not affect the long-term memory. Both ZIP and chelerythrine were equally effective at disrupting the maintenance of long-term synaptic memory in *Aplysia*. In our experiments sensorimotor cocultures, after an initial test of synaptic strength, received 5X5-HT stimulation; 24 hours later some of the cocultures were incubated for 1 hour in either ZIP or chelerythrine. The synapses were then retested at 48 hours after training. LTF was reversed at 48 hours in cocultures treated with either ZIP or chelerythrine, whereas cocultures incubated at 24 hours in either scrambled ZIP or control perfusion medium exhibited significant facilitation. Similar results have been reported by Hu et al. ¹⁶⁵ who trained cocultures with 5X5-HT for two consecutive days; such training yields LTF that persists for one week. These investigators found that treatment with chelerythrine on the second day of 5-HT treatment eliminated all LTF, causing a return of the sensorimotor EPSP to its baseline amplitude by Day 3 of the experiment; moreover, no facilitation was evident on Day 7. Treatment with the PKM inhibitor on the second day of 5-HT training also blocked the increase in presynaptic sensorin expression normally observed 24 hours

later. Taken together, the results of these two studies provide strong support for the notion that PKC activity, specifically, that of PKM Apl III, supports the long-term maintenance of memory in *Aplysia*.

Memory reconsolidation in *Aplysia*

One of the most intriguing of memory phenomena is so-called memory reconsolidation. Until relatively recently, the predominant idea regarding memory persistence has been that memories destined for long-term storage underwent a single, protein synthesis-dependent process of consolidation, after which they were stable, barring injury or disease ¹⁶⁶. Opposed to this idea have been data indicating that memories could become labile and subject to disruption upon reactivation ¹⁶⁷⁻¹⁷⁰. Recent years have witnessed increasing evidence, from studies of both vertebrates and invertebrates, of the lability of reactivated long-term memories ¹⁷¹⁻¹⁸⁰. Two studies have now demonstrated reconsolidation of long-term synaptic and behavioral memory in *Aplysia*; specifically, both Cai et al. ¹⁰⁶ and Lee et al. ¹⁸¹ showed that the memory for LTS and LTF can become disrupted by inhibition of protein synthesis following reactivation of the behavioral/synaptic memory. (A methodological difference between the two studies is that Cai and colleagues used brief sensitization training/5-HT stimulation to reactivate the memory for LTS/LTF, Lee et al. used test-type stimulation—weak tactile stimulation of the siphon in the case of LTS and brief homosynaptic activation of the synapse in the case of LTF—for memory reactivation.) The study by Hu et al. ¹⁶⁵ provides additional support for the idea that the memory for LTF can undergo reconsolidation; they found that if a

protein synthesis inhibitor was applied to sensorimotor cocultures during the second day of 5-HT training (see above), both the new facilitation, resulting from training on the second day, and the persistent facilitation, resulting from the first day of 5-HT training, were disrupted.

Lee et al.'s ¹⁸¹ results implicate the ubiquitin-proteasome pathway in memory reconsolidation in *Aplysia*. These investigators found that the ubiquitin/proteasome inhibitor β -lactone blocked the disruption of long-term behavioral and synaptic memory when β -lactone was applied together with the protein synthesis inhibitor immediately following memory reactivation. These results suggest that when the long-term memory is reactivated it is destabilized by protein degradation via the ubiquitin/proteasome pathway; subsequently—barring inhibition of protein synthesis—the memory becomes restabilized via new translation see also refs. ^{182,183}.

The data from the studies of memory “erasure” through inhibition of PKM and of memory reconsolidation in *Aplysia* have important implications. It has been contentious in the field of memory research whether, when long-term memories are apparently erased by inhibition of PKM or through disruption of reconsolidation, the memories are permanently eliminated or whether they are, instead, temporarily unable to be retrieved [see, e.g., ref. ¹⁸⁴](#). Another issue of contention is the extent to which reconsolidation of memory resembles original consolidation ^{173,175,185}. These issues are difficult to resolve in the absence of a realistic synaptic model of memory erasure and reconsolidation. The recent studies in *Aplysia* provide such a model. The cellular and molecular consequences of inhibiting PKM Apl III, or of disrupting memory reconsolidation, can be examined over several days at a single synapse in cell culture, one amenable to rigorous

mechanistic analyses. Furthermore, the results from studies of the *Aplysia* in vitro sensorimotor synapse are likely to have significant ecological validity. The extensive body of prior work on the mechanisms underlying behavioral plasticity of the defensive withdrawal reflex, some of which has been reviewed here, has established that, not only can the findings from experiments on sensorimotor cocultures be extrapolated to the in vivo synapse, but they will also prove relevant to actual learning in the intact animal. One can therefore anticipate that future studies in *Aplysia* will provide valuable insights into the processes that underlie memory erasure and reconsolidation.

Summary

Over the last four decades sensitization of the defensive withdrawal reflex of *Aplysia* has served as a valuable model system for understanding the cellular and molecular mechanisms that mediate long-term memory. Importantly, discoveries from work on LTS in *Aplysia*, such as the importance of CREB-dependent transcription, have generalized to other systems, particularly mammalian systems. Moreover, I believe that the mine of mechanistic insights into long-term memory opened by work on learning and memory in *Aplysia* is far from being exhausted. As suggested by the recent work on PKM Apl III and on memory reconsolidation, in particular, one can anticipate that studies of LTS and LTF in *Aplysia* will be a source of fundamental knowledge about how the brain stores and maintains memories for many years to come.

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FIGURES

Figure 1. Long-term facilitation of the sensorimotor synapse *Aplysia* in dissociated cell culture. **A₁**, Sensorimotor coculture. Here the presynaptic neuron is a pleural sensory neuron ²⁴ and the postsynaptic neuron is a small siphon (LFS) motor neuron ²³. Scale bar, 20 μ m. **A₂**, Sample electrophysiological records from a pretest of a synapse. Scale bars, 20 mV and 200 ms. **B₁**, Experimental protocol for the demonstration of 24 hr LTF. The coculture was treated with five spaced 5-minute applications of 5-HT (100 μ M); the drug was washed out of the cell culture for 15 minutes between 5-HT pulses. **B₂**, Sample EPSPs. Each pair of traces shows EPSPs recorded from the same sensorimotor synapse on Day 1 (Pre) and ~ 24 hr later (Post). Training with 5-HT produced LTF (lower traces). Scale bars, 10 mV and 80 ms. From ref. ¹⁴⁹.

Figure 2. Cellular model for sensitization-related long-term facilitation. Prominent features of the model are postsynaptic modulatory input from monoaminergic interneurons and retrograde signaling. Prolonged stimulation with 5-HT causes enhanced release of glutamate, the presynaptic transmitter, and modulation of postsynaptic AMPA-type receptor trafficking; the latter process involves exocytotic insertion of AMPA receptors into the cell membrane of the motor neuron. This process is mediated by G protein-stimulated release of Ca²⁺ from intracellular stores and enhanced spontaneous presynaptic release (not shown). The elevated intracellular Ca²⁺ is also responsible, either directly or indirectly (perhaps through protein synthesis), for triggering the activation of one or more retrograde signals; the retrograde signals, in turn, contribute critically to presynaptic changes and enhanced presynaptic release. Not shown are potential anterograde signals ⁶² nor processes that mediate the maintenance of LTF ^{110,149,155}. Also, both LTS and LTF involve the growth of presynaptic processes and varicosities, as well as postsynaptic spines; these structural changes are not shown. From ref. ⁵⁸